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TITLE: The Role of Neuropeptide Y (NPY) In Uncontrolled Alcohol Drinking and Relapse Behavior Resulting From Exposure to Stressful Events

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14. ABSTRACT   There is high comorbidity between post-traumatic stress disorder (PTSD) and alcohol dependence, indicating that exposure to stressful events increases the risk of alcoholism. Thus, identifying pharmacological targets with potential therapeutic value in treating PTSD-associated alcoholism is critical. An interesting candidate is neuropeptide Y (NPY). Recent evidence suggests that low NPY levels promote high alcohol consumption, and it has been established the NPY protects against stress and anxiety. The overall goal of this grant is to determine the role of NPY (and related neuropeptides) in modulating stress-induced increases of alcohol consumption using mouse models. The specific projects for the current funding year determined if A) overexpression of brain NPY with a recombinant adeno-associated virus (rAAV) vector is protective against increased alcohol consumption, and B) if mutant mice lacking normal production of NPY show enhanced sensitivity to stress-induced increases of ethanol consumption. Results indicate that overexpression of brain NPY protects against high alcohol drinking in mice, and that a lack of NPY in mutant mice increases sensitivity to stress-induced alcohol self-administration. Together, the current findings provide evidence that NPY signaling protects against the effects of stress on excessive alcohol self-administration. Thus, NPY may have therapeutic value in treating alcoholism triggered by PTSD.

15. SUBJECT TERMS
  Alcohol, Alcoholism, Ethanol, Post-Traumatic Stress Disorder, Mice, Neuropeptide Y, Relapse

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INTRODUCTION: People who have been exposed to an extremely traumatic event, such as witnessing a death, receiving a threat of death, or experiencing a serious injury, may develop a set of symptoms known as posttraumatic stress disorder (PTSD). Events that contribute to the development of PTSD are common to individuals placed in a combat environment. Evidence suggests that there is a high comorbidity between PTSD and alcohol dependence. Given the prevalence of PTSD among veterans of war and the increased risk of alcoholism for individuals suffering from PTSD, identifying pharmacological targets with potential therapeutic value in treating PTSD-associated alcoholism may be considered of high relevance to the U.S. military. An interesting candidate is neuropeptide Y (NPY), and neurochemical that is present throughout the central nervous system. NPY is involved with a diverse set of biological functions including the integration of emotional behavior such as anxiety and depression. Interestingly, recent evidence suggests that low NPY levels and deletion of NPY or the NPY Y1 receptor promote high alcohol consumption. Furthermore, combat-related PTSD is associated with decreased plasma levels of NPY, and uncontrolled stress caused by exposure to military survival training results in depletion of plasma NPY levels following extended exposure. Because low NPY levels promote increased alcohol intake, reduced NPY associated with PTSD may be a factor that leaves individuals susceptible to alcoholism. **Therefore, the guiding hypothesis of the present proposal is that normal NPY signaling protects against uncontrolled alcohol drinking and relapse caused by exposure to stressful events.** To address this issue, a set of studies have been proposed using animal models of stress-induced alcohol consumption. Mutant mice lacking normal NPY signaling, and overexpression of NPY with the use of a recombinant adeno-associated virus (rAAV) vector that causes expression and constitutive secretion of NPY (rAAV-FIB-NPY), are powerful tools used in the present research. These studies will establish if normal NPY signaling protects against the effects of stress on uncontrolled alcohol drinking and relapse of ethanol-seeking behavior.

BODY: The experiments described below fall into 2 categories: Those related to Tasks 3 and 4 of the Statement of Work, and additional experiments that were run which complement work that is outlined in this proposal and which are useful for the completion of the additional Tasks in future funding years. We tackled Tasks 3 and 4 first as procedures involved in these Tasks are currently being used in our lab. Thus, for logistical reasons it made most sense to begin with Tasks 3 and 4.

TASK 3: ADENO-ASSOCIATED NPY VIRAL VECTOR TRASDUCTION IN THE NUCLEUS ACCUMBENS AND AMYGDALA ALTERS ETHANOL SENSITIVITY IN MICE. Accumulating genetic and pharmacological evidence suggest that neuropeptide Y (NPY) modulates neurobiological responses to ethanol. We recently found that the high ethanol drinking C57BL/6J inbred strain of mice have significantly lower NPY expression in the amygdala and nucleus accumbens (NAc) when compared to the ethanol avoiding DBA/2J inbred strain (Hayes et al., 2005). To determine if low NPY expression contributes to altered neurobiological responses to ethanol that are characteristic of C57BL/6J mice, we used a recombinant adeno-associated viral (rAAV) vector that promotes production and secretion of NPY in transduced cells. In one rAAV vector, the coding sequence for NPY was preceded by the signal sequence for the laminar protein, fibronectin (rAAV-FIB-NPY), because inclusion of this FIB secretory sequence leads to the secretion of NPY from transduced cells. Additionally, a rAAV-NPY vector and a control vector with the coding sequence for green fluorescent protein (rAAV-GFP) were used. In two separate experiments, vectors were infused into the region of the amygdala (1.0 μl/10-min) or the core of the NAc (0.5 μl/10-min) of male C57BL/6J mice. Animals rested for two weeks following surgery to allow for adequate gene expression and secretion. Following amygdala infusion, mice showed normal baseline ethanol consumption. However, relative to

rAAV-GFP and rAAV-NPY treated animals, mice treated with the rAAV-FIB-NPY vector drank significantly less ethanol for up to 24-h when ethanol was returned following two weeks of imposed ethanol abstinence (**Figure 1**). On the other hand, while transduction of the vectors into the core of the NAc did not alter baseline ethanol drinking or consumption following a two week ethanol deprivation period, both the rAAV-NPY and rAAV-FIB-NPY vectors protected against the locomotor stimulant effects caused by intraperitoneal injection of a 1.5 g/kg dose of ethanol over a 5-day period (one ethanol injection per day; **Figure 2**). These observations indicate that the effects of NPY on neurobiological responses to ethanol are brain-region specific. We are currently determining if transduction of the rAAV-FIB-NPY vector in the amygdala will protect against further increases of ethanol drinking caused by exposure to foot-shock stress during the ethanol abstinence period.

TASK 4: LACK OF NPY IN GENETICALLY ALTERED MICE LEAVES ANIMALS MORE SENSITIVE TO THE EFFECTS OF STRESS ON RELAPSE OF ETHANOL-SEEKING BEHAVIOR: Alcoholism is a chronic medical condition involving periods of relapse. Clinical data suggest that exposure to stressful life events is a critical factor contributing to relapse of alcoholism after abstinence. The purpose of the present experiment was to explore the effect of foot-shock stress on reinstatement of ethanol-seeking behavior (i.e., pressing a lever that had previously been reinforced with ethanol) after lever responding behavior was extinguished (i.e., lever pressing was not reinforced with ethanol over multiple trials). This procedure is an accepted animal model of relapse. Mutant mice lacking NPY (NPY-/-) or normal wild-type mice (NPY+/+) were trained (1-hr sessions) in operant chambers that contained two levers: an active lever reinforced with 14% ethanol and an inactive lever to track non-specific responding. After stable ethanol responding, mice were trained under an extinction paradigm where lever pressing did not result in ethanol delivery. Lever-pressing behavior was monitored until no significant differences existed between the ethanol and inactive lever responses for 3 consecutive days. Following the extinction phase, half of the mice were exposed to foot shocks with an intensity of 0.5 mA (0.5 second duration, a mean 40 second inter-shock interval) for a period of 10 minutes. The remaining mice were placed in the shock apparatus but not given shocks. Immediately following exposure to foot shocks, mice were placed back in the operant chambers and given access to the ethanol and water levers. Results indicated that when mice were exposed to foot-shock, NPY-/- mice showed reinstatement of ethanol lever responding reflected by significantly more responses to the ethanol lever relative to the inactive lever (See **Figure 3**). On the other hand, NPY+/+ did not show differential responding between the ethanol and inactive levers, indicating that shock-shock did not reinstate ethanol-seeking behavior in mice with normal NPY production. There was not reinstatement of ethanol-seeking behavior in either NPY-/- or NPY+/+ mice not exposed to shock (data not shown). These results indicate that normal NPY signaling is protective against the effects of stress on relapse of ethanol-seeking behavior.

ADDITIONAL RELATED RESEARCH: A CRF-1 RECEPTOR ANTAGONIST PROTECTS AGAINST EXCESSIVE ETHANOL SELF-ADMINISTRATON CAUSED BY ETHANOL RELAPSE: A transient increase of ethanol self-administration following interruption of ethanol access is called the alcohol deprivation effect (ADE) and is one animal model of excessive ethanol drinking that is characteristic of relapse. The neurochemical substrate that modulates this phenomenon is not well characterized. To determine if increased ethanol-reinforced lever pressing resulting from interruptions of operant procedures is modulated by the corticotropin releasing factor-1 (CRF-1) receptor, C57BL/6J mice were trained in a 2-hour operant self-administration paradigm to lever press for 10% ethanol or water on separate response keys. Following baseline, mice were given a break from the operant procedures for 4-days, and were then retested with ethanol and water in the operant paradigm for 3 consecutive days. This

interruption of operant procedures was repeated over multiple cycles. Following the fifth cycle, mice were given intraperitoneal (i.p.) injection (0, 10, or 20 mg/kg) of the CRF-1 receptor antagonist, CP-154,526, 30-minutes before testing. Results indicated that mice exhibited significant increases of lever presses reinforced by ethanol following 4-day interruptions of procedures relative to pre-interruption baseline (BL) responding (**Figure 4**). Furthermore, the increase of lever pressing following the 4th 4-day break from operant procedures was significantly greater than after the 1st break from procedures. Interestingly, i.p. injection of a 10 mg/kg dose of CP-154,526 protected against increased lever pressing resulting from a 4-day interruption of operant procedures (**Figure 5**). Thus, ethanol lever responses were similar to BL responding following the 10 mg/kg dose of the CRF-1 receptor antagonist. These findings indicate that the increased motivation to perform ethanol-reinforced responding resulting from interrupting operant procedures is modulated by the CRF-1 receptor. Thus, the CRF-1 receptor, known to modulate stress responses, may also modulate excessive ethanol drinking following periods of abstinence.

ADDITIONAL RELATED RESEARCH: A CRF-1 RECEPTOR ANTAGONIST PROTECTS AGAINST INCREASED ETHANOL DRINKING RESULTING FROM EXPOSURE TO A STRESSOR: Evidence from human and animal research indicate that exposure to stress increases the amount of ethanol consumed. For the present project, we determined if the stress-related neuropeptide, CRF, modulates increased ethanol drinking resulting from exposure to stress. Individually housed Balb/cJ mice were given free access to 8% ethanol in one bottle and water in a second bottle with *ad libitum* access to food. Once ethanol drinking stabilized, half the mice were exposed to a forced-swim stressor for 5 minutes, once a day for 5 days, and the remaining mice were only handled (no stress condition). Within each condition (stress or no stress), half the animals were given i.p. injection of a 10 mg/kg dose of CP-154,526 (the CRF-1 receptor antagonist) before each stress or handling trial. The remaining mice were given injection of a vehicle. Results indicated that exposure to forced-swim stress caused a 4-fold increase in ethanol drinking 2-3 weeks following the stress procedures in the stress/vehicle treated group of mice (**Figure 6**). However, pre-treatment with the CRF-1 receptor antagonist blocked stress-induced increases of ethanol drinking in the stress/CRF-1 receptor antagonist treated group. These findings provide direct evidence that the stress-induced increase of ethanol drinking is modulated by the stress-related neuropeptide, CRF. These procedures will now be used to further assess the role of NPY in modulating stress-induced increases of ethanol drinking.

KEY RESEARCH ACCOMPLISHMENTS: A list of key research accomplishment achieved during the first budget year of this grant are as follows:

- Establishing that overexpression of NPY (via the rAAV-FIB-NPY vector) in the central nucleus of the amygdala protects against excessive relapse ethanol drinking.

- Establishing that overexpression of NPY in the nucleus accumbens with the rAAV-FIB-NPY vector protects against the locomotor stimulant effects of ethanol.

- Establishing that a lack of NPY in genetically altered mice leaves animals more sensitive to the effects of stress on relapse of ethanol-seeking behavior.

- Establishing that an antagonist for another stress-related neuropeptide called corticotropin releasing factor (CRF) protects against excessive ethanol self-administration caused by abstinence from ethanol.

- Establishing that the CRF receptor antagonist protects against increased ethanol drinking resulting from exposure to a stressor.

REPORTABLE OUTCOMES: The following is a list of what has been supported by this grant during the first budget year:

PUBLICATON

Sparta, D. R., Ferraro, F. M., Fee, J. R. Knapp, D. J., Breese, G. R., & Thiele, T. E. (under review). Repeated interruptions of operant procedures strengthens ethanol and water reinforced responding in C57BL/6J mice: Modulation by CRF-1 receptors. *Psychopharmacology.*

CONFERENCE PRESENTATIONS

Thiele, T. E., Hayes, D. M., Knapp, D. J., Breese, G. R., & McCown, T. J. (2006). Adeno-associated NPY viral vector transduction in the nucleus accumbens and amygdala alters ethanol sensitivity in mice. Presented at the 8th NPY meeting in St. Petersburg, FL.

Thiele, T. E., Hayes, D. M., Knapp, D. J., Breese, G. R., & McCown, T. J. (2006). Central adeno-associated NPY viral vector transduction alters ethanol sensitivity in mice. Presented at the annual meeting of the Research Society on Alcoholism, Baltimore, MD.

CONCLUSIONS: We have made significant progress towards the goals of this research proposal. We have demonstrated that overexpression of NPY with a viral vector in the amygdala of mouse brain protects against increased ethanol drinking resulting from abstinence from ethanol. Over expression of NPY in the nucleus accumbens protects against the stimulant effects of ethanol. Importantly, we show that a lack of NPY in mutant mice leaves animals susceptible to stress-induced relapse of ethanol-seeking behavior. Additional research demonstrates that another stress-related neuropeptide, CRF, modulates stress-induced increases of ethanol drinking in mice, and also modulates increases of ethanol drinking resulting from ethanol abstinence. So what does this mean? These results have important implications for possible pharmacological medical treatment of stress-related alcoholism and alcohol relapse. Pharmacological targets aimed at the NPY and CRF systems may prove to be effective in treating alcoholism resulting from exposure to traumatic events and stemming from PTSD. Thus, these findings may be considered of high relevance to the U.S. military.

REFERENCE:

Hayes, D. M., Knapp, D. J., Breese, G. R., & Thiele, T. E. (2005). Comparison of basal NPY and CRF levels between the high ethanol drinking C57BL/6J and low ethanol drinking DBA/2J inbred mouse strains. *Alcoholism: Clinical & Experimental Research, 29,* 721-729.

APPENDICES:

- Figures 1-6. In figures, * indicates significant differences between groups at the $p < 0.05$ level.

- A pre-print of our paper cited in the Reportable Outcomes section above. This paper is currently under review for the journal *Psychopharmacology.*
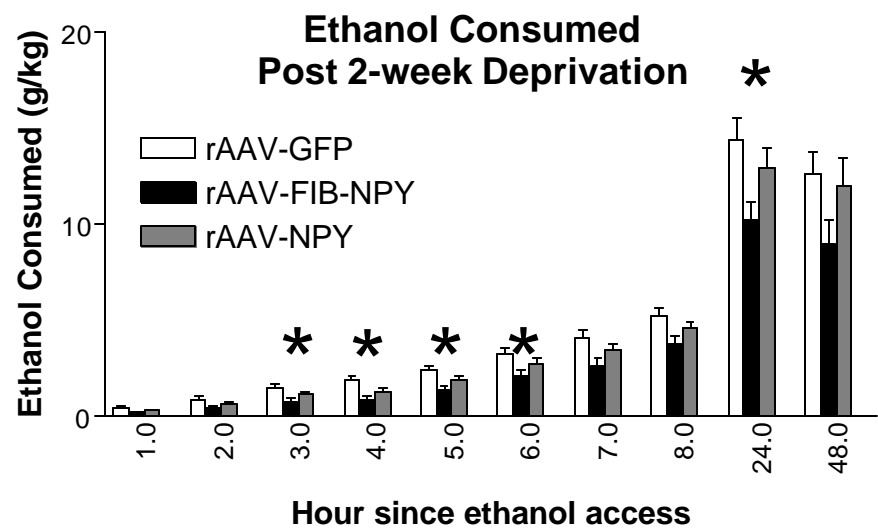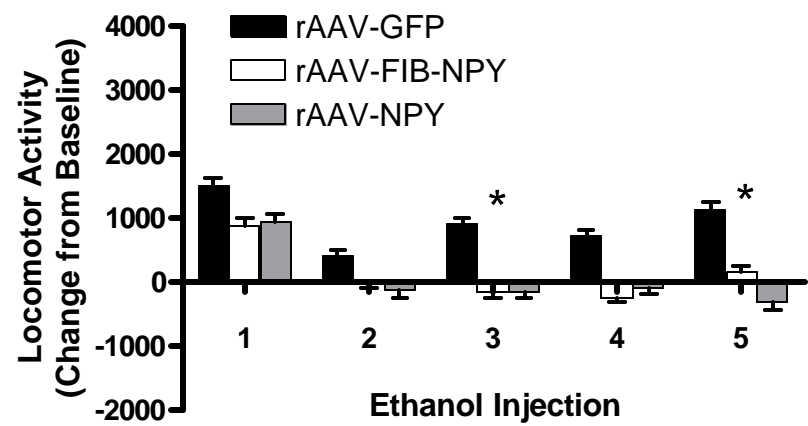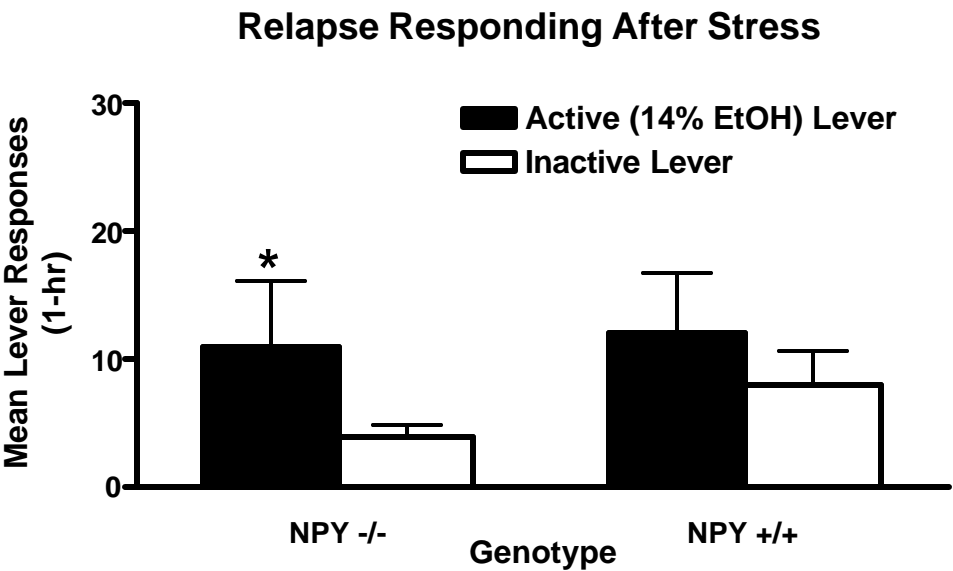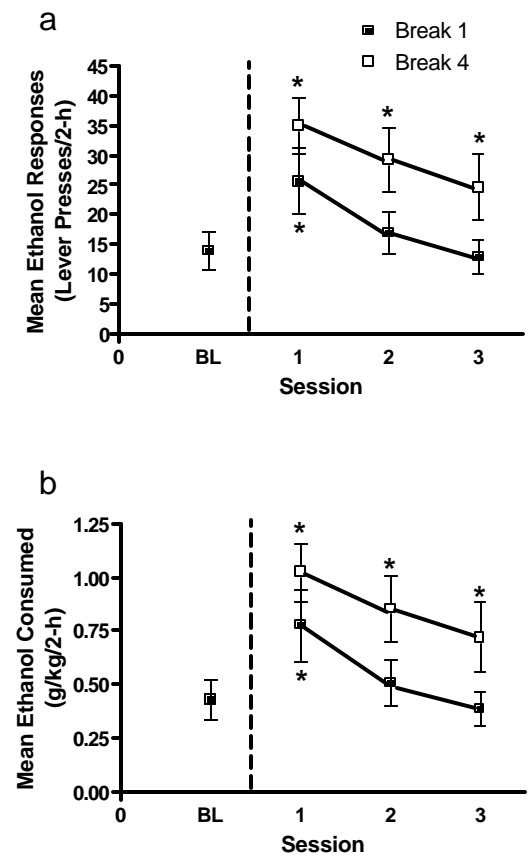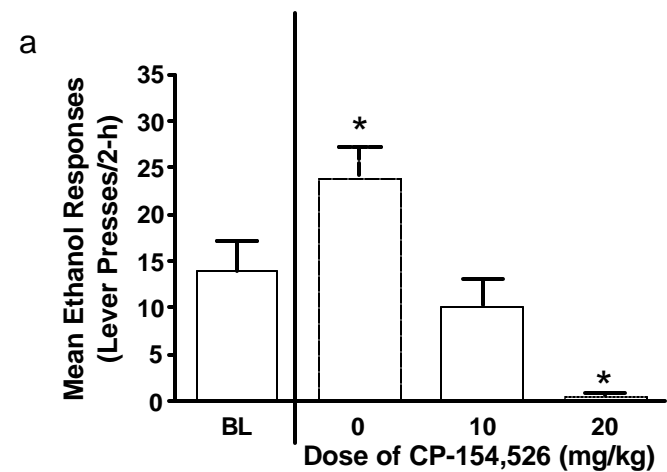
Figure 1

**Ethanol Consumed
Post 2-week Deprivation**

rAAV-GFP
rAAV-FIB-NPY
rAAV-NPY

Ethanol Consumed (g/kg)

Hour since ethanol access

Figure 2

rAAV-GFP
rAAV-FIB-NPY
rAAV-NPY

Locomotor Activity
(Change from Baseline)

Ethanol Injection

Figure 3

Relapse Responding After Stress

Mean Lever Responses (1-hr)

- Active (14% EtOH) Lever
- Inactive Lever

Genotype: NPY -/-, NPY +/+

Figure 4

a

Mean Ethanol Responses (Lever Presses/2-h)

- Break 1
- Break 4

Session

b

Mean Ethanol Consumed (g/kg/2-h)

Session

Figure 5

a

Figure 6

Repeated interruptions of operant procedures strengthens ethanol and water reinforced responding in C57BL/6J mice: Modulation by CRF-1 receptors

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ABSTRACT

Rationale: A transient increase of ethanol self-administration observed following interruption of ethanol access is frequently referred to as the alcohol deprivation effect (ADE). The neurochemical substrate that modulates this increased ethanol drinking is not well characterized. *Objectives:* To determine if an observed increase in ethanol-reinforced lever pressing resulting from interruptions of operant procedures is modulated by the corticotropin releasing factor-1 (CRF-1) receptor. *Methods:* C57BL/6J mice were trained in a 2-hour operant self-administration paradigm to lever press for 10% ethanol or water on separate response keys. Once stable responding occurred, mice were given a break from the operant procedures for 4-days, and were then retested with ethanol and water in the operant paradigm for 3 consecutive days. This interruption of operant procedures was repeated over multiple cycles. Following the fifth cycle, mice were given intraperitoneal (i.p.) injection (0, 10, or 20 mg/kg) of the CRF-1 receptor antagonist, CP-154,526, 30-minutes before testing. The effects of CP-154,526 in mice not experiencing interruptions of their sucrose- or water-reinforced lever pressing was also determined. *Results:* Mice exhibited significant increases of lever presses reinforced by ethanol or water following 4-day interruptions of operant procedures. Interestingly, i.p. injection of a 10 mg/kg dose of CP-154,526 protected against increased lever pressing without altering baseline self-administration of water or a sucrose solution. *Conclusions:* Increased motivation to perform ethanol- and water-reinforced responding resulting from interrupting operant procedures is modulated by the CRF-1 receptor. These results, which may not represent an ADE, are best explained by a time-dependent increase of the incentive motivation elicited by contextual conditioned stimuli resulting from interruptions of operant procedures.

Key Words: C57BL/6J Mice, Alcohol Deprivation Effect (ADE), Incubation, Operant Self-Administration, Corticotropin Releasing Factor

INTRODUCTION

Alcohol relapse is a major problem in the treatment of alcoholism. Approximately 60-80% of abstinent alcoholics will relapse at one point in their lifetime (Barrick and Connors 2002; Chiauzzi 1991). Thus, understanding the neurobiology of relapse and associated behaviors is a critical step towards the development of drugs aimed at treating alcoholism. Relapse after long periods of abstinence is frequently associated with significant increases in the amount of ethanol consumed relative to pre-abstinence levels (Holter et al. 2000). Recent procedures have been developed and validated as animal models of relapse-associated behaviors. One procedure involves periodic removal of ethanol self-administration procedures and/or ethanol after which animals self-administer significantly more ethanol than they had consumed prior to the break in ethanol self-administration. This phenomenon has been labeled the alcohol deprivation effect (ADE) and is thought to model compulsive uncontrolled relapse drinking characteristic of alcohol dependent humans (Spanagel and Holter 1999). The ADE is a robust phenomenon evident in rats (Heyser et al. 1997; McKinzie et al. 1998; Rodd et al. 2003; Rodd-Henricks et al. 2001; 2002a; b; Wolffgramm and Heyne 1995), monkeys (Kornet et al. 1990; Sinclair 1971) and humans (Burish et al. 1981; Mello 1972).

Here we sought to further characterize the neurochemical substrate involved in modulating increased ethanol self-administration resulting from interruptions of ethanol self-administration procedures. To date, the glutamate (Backstrom et al. 2004; Holter and Spanagel 1999; Sanchis-Segura et al. 2006; Spanagel et al. 1996; Vengeliene et al. 2005) and opioid (Holter et al. 2000) systems have been implicated in modulating deprivation-induced increases of ethanol drinking. An additional target of interest is corticotropin releasing factor (CRF), a 41 amino acid polypeptide with high concentrations in the hypothalamus, the brainstem, and the amygdala (Swanson et al. 1983). A role for CRF in modulating neurobiological responses to ethanol has been well established. Both acute and chronic ethanol exposure activate central

CRF (Koob et al. 1993; Rasmussen et al. 2000; Rivier et al. 1984), and the anxiogenic effect of ethanol withdrawal is reversed by CRF receptor antagonists (Breese et al. 2004; Knapp et al. 2004; Overstreet et al. 2004; Rassnick et al. 1993). Central infusion of the CRF receptor antagonist, D-Phe-CRF$_{(12-14)}$, eliminates excessive ethanol drinking by rats made dependent with chronic exposure to ethanol vapor (Valdez et al. 2002). Additionally, stress-induced reinstatement of operant ethanol self-administration is blocked after administration of a CRF receptor antagonist and increased by central infusion of CRF (Le et al. 2000; Stewart 2004). Given the established role of CRF in modulating neurobiological responses to ethanol, we sought to determine if CRF-1 receptor signaling contributes to increased ethanol self-administration resulting from interruptions of self-administration procedures.

To address this question, we studied ethanol self-administration by C57BL/6J mice following administration of the CRF-1 receptor antagonist, CP-154,526. C57BL/6J mice are one of the most commonly used inbred strains of mice for studying neurobiological responses to ethanol as they exhibit high ethanol consumption (>10 g/kg per day) (Belknap et al. 1993) and can achieve pharmacologically relevant blood ethanol concentrations during limited ethanol access (> 100 mg/ml after 4-hours of access) (Rhodes et al. 2005). Because significant increases of ethanol-reinforced lever pressing following forced interruptions of operant procedures has been observed in C57BL/6J mice (Middaugh et al. 2000a), we used an operant paradigm in the present set of studies. Our data show that interrupting operant procedures promotes increased lever presses reinforced by ethanol or water, and that increased reinforced responding is modulated by CRF-1 receptor signaling. These results may not represent a true ADE are best explained by a time-dependent increase of the incentive motivation elicited by contextual conditioned stimuli resulting from interruptions of operant procedures.

METHODS

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor ME) were used in all experiments. Mice were 6-8 weeks old, weighed between 25-30 g at the start of all experiments and were single housed in polypropylene cages with corncob bedding and *ad libitum* access to food and water. Standard rodent chow (Teklad, Madison, WI) and water were available at all times except where noted. The vivarium rooms were maintained at an ambient temperature of 22^0 C with a 12- hour/12-hour light-dark cycle. All experimental procedures were approved by the University of North Carolina Animal Care and Use Committee (IACUC) and complied with the NIH Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

Drugs

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was donated by Pfizer (Groton, CT), and was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF-1 receptor ($K_i < 10$ nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al. 1996; Schulz et al. 1996). Importantly, systemic injection of CP-154,526 blocks anxiety-like behavior stemming from ethanol withdrawal in rats (Breese et al. 2005). During operant training, mice received daily intraperitoneal (i.p.) injections of 0.5% CMC (5 ml/kg) 30-minutes before operant sessions to habituate them to injection procedures (except where noted). Injection site was alternated between sides (left or right) daily to minimize tissue damage.

Operant Ethanol and Water Self-Administration following Interruptions of Operant Procedures

Operant self-administration experiments were conducted in sixteen modular mouse operant chambers (Med Associates, Georgia, VT) with dimensions of 21.6 x 17.8 x 12.7 cm and

a stainless steel grid floor. All chambers were housed in a sound-attenuating shell with a ventilation fan. Liquid receptacles with nose-poke sensors were located in the center of the right and left chamber walls and a stainless steel response lever was to the right of each receptacle. Liquid solutions (one lever produced water, the second lever produced sucrose or ethanol) were infused using 10 ml plastic syringes which were mounted on a programmable pump (PHM-100, 3.33 rpm). The pump delivered 0.01 ml of solution per activation. A yellow stimulus light and tone (80 dB) were activated when the sucrose/ethanol response lever was depressed. No stimulus light or tone occurred when the water lever was pressed. A single standard food pellet was placed inside the operant chamber during 2-hour test sessions. A house light inside the operant chambers was turned on for the duration of the test. Data recorded during each session included the number of sucrose/ethanol and water responses (bar presses), the number of sucrose/ethanol and water reinforcers (pump activation), and ethanol intake (g/kg body weight). The operant chambers were interfaced to an IBM computer and all data were automatically recorded using Med Associates software (MED-PC for Windows®, Version IV). All operant sessions were completed in the light phase of the light/dark cycle.

All mice (n=32) were placed under a modified operant sucrose fading procedure (Samson 1986; Schroeder et al. 2003). Briefly, mice were initially trained to press two operant levers. In all operant procedures described in this report, mice were placed on a fixed ratio (FR)-1 schedule of reinforcement. One lever resulted in the delivery of a 10% sucrose solution (w/v), the other lever delivered distilled water. Mice were allowed to respond for 10% sucrose or water for 4-days in 16-hour sessions in order to strengthen lever pressing behavior. Sessions were then reduced to 2-hours per day for 4-days. At this point and for the remainder of the experiment, mice were given access to two bottles in their homecages (one containing water and the other containing an ethanol solution). The ethanol concentration presented in the homecage matched the concentration of ethanol being tested in the operant chambers. Thus,

animals had access to ethanol for 24-hours per day during the ethanol training phase. We gave mice access to ethanol in their homecage so that the only time they would be deprived of ethanol was during the planned breaks from operant procedures (see below). Following stable responding, increasing concentrations of ethanol were introduced to the 10% sucrose solution every 2-days (2, 4, 8, and 10% ethanol (v/v)). Then, the sucrose concentration was reduced every 2-days (5, 2, and 0% sucrose) until mice were responding only for 10% ethanol.

Once mice displayed stable responding for 10% ethanol (8 sessions), mice were given a break from operant procedures and homecage ethanol was removed for 4-days. Mice were then tested in 2-hour operant sessions over 3 consecutive days and they were again given access to 10% ethanol in their homecages along with food and water. Following the third operant session, mice were again given a 4-day interruption of operant procedures/homecage ethanol access and then given access to ethanol in the operant chambers and homecage for 3 consecutive days. This procedure involving 3-days of operant procedures followed by a 4-day interruption of procedures was repeated for a total of 4-cycles.

Effect of CP-154,526 on Operant Responding

Mice continued cycles of ethanol access (3-days) and interruptions of operant procedures (4-days) as described above. In the procedures below, mice were administered CP-154,526 in a counterbalanced order. Immediately following the fifth, 4-day interruption of operant procedures, mice were injected with one of three doses of CP-154,526 (0, 10, 20 mg/kg) mixed in 0.5% CMC 30-minutes prior to the start of the first operant session. Immediately following the seventh, 4-day interruption of operant procedures, mice were injected with one of two doses of CP-154,525 (0 or 10 mg/kg). We chose not to use the 20 mg/kg dose of CP-154,526 during the second administration because this dose suppressed ethanol and water self-administration below baseline levels.

Open-Field Locomotor Activity after i.p. Injection of CP-154,526

To determine whether CP-154,526 could impair motor activity, naïve mice were tested in an open-field arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA). The open field arena measured 40.64 cm by 40.64 cm by 30.48 cm and was made of clear Plexiglas. Several cm of corncob bedding were placed into the open field chamber to aid in cleaning and to prevent the buildup of odor. C57BL/6J mice were handled and injected with CMC daily for 7 days before activity testing. CMC or CP-154,526 (10 mg/kg) was administered to mice (n = 10/group) and then 30 minutes later mice were placed in the center of the locomotor activity chamber. Horizontal distance traveled (in meters) was recorded as an index of motor function during a 2-hour test session.

Operant Self-Administration of Sucrose and Water following i.p. Injection of CP-154,526

To determine if a 10 mg/kg dose of CP-154,526 had a general suppressive effect on lever pressing behavior, naïve male C57BL/6J mice (n = 10) were trained to press two operant levers, one lever resulted in the delivery of a 10% sucrose solution (w/v), the other lever delivered distilled water. Mice did not experience interruptions of operant procedures. Mice were allowed to respond for 10% sucrose and water until responses stabilized. On the test day, mice were injected with 0.5% CMC or CP-154,526 (10 mg/kg) mixed in 0.5% CMC 30-minutes prior to the start of the 2-hour self-administration test.

Operant Ethanol and Water Self-Administration following an Interruption of Operant Procedures in Mice not Given i.p. Injections

To determine if the stress associated with daily i.p. injections may have altered the overall level of lever pressing, mice were tested in the operant self-administration paradigm in the absence of i.p. injections. Briefly, naïve male C57BL/6J mice (n = 32) were trained to press levers for ethanol or water reinforcement as described above except i.p. injections were never

administered. Once stable responding occurred for the 10% ethanol solution, mice were not run in the operant chambers and homecage ethanol was removed for a 4-day break. Mice were then tested in 2-hour operant sessions over 3 consecutive days and they were again given access to 10% ethanol in their homecages along with food and water.

Operant Water Self-Administration following an Interruption of Operant Procedures

To determine if increased lever pressing resulting from 4-day interruptions of operant procedures required the presence of ethanol, naïve male C57BL/6J mice (n = 11) were trained to press a lever for water reinforcement during a 2-hour session as described above. The second lever was inactive such that responses were not reinforced and no light or sound was associated with activation of the second key. Once stable responding occurred for water (about one week), mice were not run in the operant chambers for a 4-day break. Mice were then tested in 2-hour operant sessions over 3 consecutive days.

Data Analysis

All data in this report are presented as means $\pm$ SEM. We used analyses of variance (ANOVA) to analyze data from each experiment. For studies that examined lever responding over days, repeated-measures ANOVAs were used to analyze the data. When significant effects were obtained, we performed planned comparisons with paired (for repeated measures) or independent t-tests (Winer et al. 1991). Significance was accepted at $p < 0.05$ (two-tailed).

RESULTS

Operant Ethanol and Water Self-Administration following Interruptions of Operant Procedures

Fig. 1a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice at baseline (last three sessions before the first 4-day break from operant procedures) and the three sessions immediately after the first and fourth break from operant procedures (for the purpose of clarity, data following breaks 2 and 3 are not presented). A two-

way mixed-factor ANOVA performed on 10% ethanol lever response data indicated a significant main effect of session [$F(3,186) = 22.42$, $p < 0.01$] and a significant session x break interaction [$F(3,186) = 3.80$, $p = 0.01$]. Following the 1st break from operant procedures, planned comparisons revealed that mice performed significantly more responses for 10% ethanol on the first post-break session relative to baseline ethanol lever responding [$t = -3.49$, $p < 0.01$] Following the fourth 4-day break, the rate of ethanol lever pressing on the first, second, and third post-break sessions were significantly higher when compared to the baseline ethanol lever responding [$t = -6.68$, $p < 0.01$; $t = -4.24$, $p < 0.01$; $t = -3.17$, $p = 0.03$, respectively]. Mean lever responses for water at baseline and during the 2-hour sessions are shown in Fig. 1b. A two-way mixed-factor ANOVA performed on water data indicated a significant main effect of session [$F(3,186) = 16.95$, $p < 0.01$] and a significant session x break interaction [$F(3,186) = 5.24$, $p < 0.01$]. Following the 1st break, planned comparisons revealed that water lever pressing on the first session of operant testing was significantly higher than the baseline water response rate [$t = -4.67$, $p < 0.01$]. Following the 4th break, lever pressing for water on the first, second, and third post-break sessions were significantly higher when compared to the baseline water lever responding [$t = -4.96$, $p < 0.01$; $t = -2.98$, $p = 0.01$; $t = -2.82$, $p = 0.01$, respectively].

Figs. 1c and d present the amount of ethanol (g/kg) and water (ml/kg) consumed, respectively. A two-way mixed-factor ANOVA run on ethanol consumption data revealed a significant main effect of session [$F(3,186) = 21.11$, $p < 0.01$] and a significant session x break interaction [$F(3,186) = 3.38$, $p = 0.02$]. Following the 1st break from operant procedures, mice consumed significantly more ethanol relative to baseline during the first post-break session [$t = -3.39$, $p < 0.01$]. Following the 4th break, mice consumed more ethanol relative to baseline during each of the three post-break sessions [$t = -6.34$, $p < 0.01$; $t = -4.07$, $p < 0.01$; $t = -2.90$, $p < 0.01$]. Similarly, a two-way mixed-factor ANOVA run on water consumption data revealed a significant main effect of session [$F(3,186) = 16.81$, $p < 0.01$] and a significant session x break interaction

[$F(3,186) = 4.81$, $p < 0.01$]. Following the first break from training, mice showed elevated water consumption relative to baseline during the first post-break session [$t = -4.66$, $p < 0.01$], and following the 4th break from training water consumption was significantly elevated above baseline levels during each of the three sessions [$t = -4.48$, $p < 0.01$; $t = -2.76$, $p = 0.01$; $t = -2.55$, $p = 0.02$].

Effect of CP-154,526 on Operant Responding

Because analyses revealed that the counterbalanced order in which mice received the 0 or 10 mg/kg doses of CP-154,526 did not differentially influence operant behavior, the data for each dose were collapsed for the analyses below. Fig. 2a depicts the mean lever responses for 10% ethanol (2-hour session) performed by C57BL/6J mice during baseline and on the first post-break session in which mice were administered CP-154,526 (0, 10, 20 mg/kg) 30-minutes before operant testing. A one-way ANOVA comparing each of the four conditions was significant [$F(3,93) = 6.044$, $p = 0.001$]. Consistent with the ADE, mice showed significantly greater post-break lever responding following administration of the vehicle when compared to their baseline ethanol lever response rate [$t = -2.07$, $p = 0.044$]. Importantly, there was no significant difference between baseline ethanol responding and post-break ethanol responding when mice were administered the 10 mg/kg dose of CP-154,526 [$t = 0.933$, $p = 0.355$]. However, the 20 mg/kg dose of CP-154,526 significantly reduced 10% ethanol lever responding relative to baseline [$t = 2.458$, $p = 0.018$]. Fig. 2b depicts the mean lever responses for water during baseline and on the first post-break session following administration of CP-154,526 (0, 10, 20 mg/kg). A one-way ANOVA run on the data was significant [$F(3,93) = 4.94$, $p = 0.003$]. The vehicle treated group had a significantly greater number of water lever responses when compared to the baseline water lever response rate [$t = -2.18$, $p = 0.034$]. Relative to baseline, there was no significant difference in water responding following treatment with the 10 mg/kg dose of CP-154,526 [$t = 0.258$, $p = 0.79$]. However, the 20 mg/kg dose of CP-154,526 significantly reduced water lever

responding relative to baseline [t = 3.424, p = 0.001]. Importantly, the 10 mg/kg dose of CP-154,526 did not significantly reduce open-field locomotor activity (15761 $\pm$ 1614 meters/2-hours) relative to mice treated with CMC (16381 $\pm$ 1343 meters/2-hours) [t = 1.041, p = 0.386].

Operant Self-Administration of Sucrose and Water following i.p. Injection of CP-154,526

Figure 3a depicts the mean lever responses for 10% sucrose (2-hour session) performed by C57BL/6J mice following administration of CMC or CP-154,526 (10 mg/kg) 30-minutes before self-administration testing. An independent t-test revealed no significant effect of the 10 mg/kg dose of CP-154,526 on sucrose responding [t = -0.558, p = 0.586]. Additionally, the 10 mg/kg dose of CP-154,526 did not significantly alter water responding relative to the control injection [t = -1.095, p = 0.298] (Fig 3b).

Operant Ethanol and Water Self-Administration following an Interruption of Operant Procedures in Mice not Given i.p. Injections

Fig. 4a depicts the mean lever responses reinforced by 10% ethanol (2-hour session) performed by C57BL/6J mice at baseline (last three sessions before the 4-day break from operant procedures) and the three sessions of post-break responding. Following the break from training, mice performed significantly more responses for 10% ethanol relative to baseline during the first post-break session [t = -4.49, p < 0.01]. Mean lever responses reinforced by water at baseline and during the 2-hour post-break sessions are shown in Fig. 4b. There were significantly more lever responses reinforced by water on the first post-break session relative to baseline levels [t = -3.96, p = 0.01]. Figs. 4c and d present the amount of ethanol (g/kg) and water (ml/kg) consumed by mice, respectively. Following the break from operant procedures, mice consumed significantly more ethanol relative to baseline during the first post-break session [t = -4.07, p < 0.01]. Similarly, mice consumed significantly more water relative to baseline during the first post-break session [t = -3.34, p = 0.02].

Operant Water Self-Administration following an Interruption of Operant Procedures

Figure 5a depicts the mean lever responses for water (2-hour session) performed by C57BL/6J mice at baseline (last three sessions before the break from operant procedures) and the three post-break sessions. Analyses revealed that mice performed significantly more responses for water on the first [$t = -3.59$, $p = 0.005$] and third [$t = -2.81$, $p < 0.018$] post-break sessions relative to baseline water-reinforced lever responding. Mean lever responses on the inactive lever at baseline and during the three post-break sessions are shown in Fig. 5b. There were no significant difference in inactive lever responses at any of the three post-break sessions relative to baseline.

DISCUSSION

There are several significant observations in the present report. First, we found that male C57BL/6J mice exhibit significant increases of ethanol- and water-reinforced lever pressing following 4-day interruptions of operant procedures. Furthermore, multiple interruptions increased the magnitude and duration of strengthened reinforced responding (Fig. 1). A second important observation is the pre-treatment with a 10 mg/kg dose of the CRF-1 receptor antagonist, CP-154,526, 30-minutes before testing protected against the expression of increased ethanol- and water-reinforced responding (Fig. 2). These findings provide novel evidence that the expression of strengthened reinforced lever pressing resulting from interruptions of operant procedures is modulated by CRF-1 receptor signaling. The 10 mg/kg dose of CP-154,526 did not reduce ethanol or water lever responding below baseline levels or significantly alter open-field locomotor activity. Additionally, this dose of the CRF receptor antagonist did not alter lever presses reinforced by sucrose or water in mice that did not experience interruptions of operant procedures (Fig. 3). Taken together, these observations limit the likelihood that the ability of the 10 mg/kg dose of CP-154,526 to reduce elevated operant

behavior resulting from interruptions of operant procedures was related to non-specific side-effects. However, the 20 mg/kg dose of CP-154,526 may have produced non-specific effects as this dose reduced ethanol- and water-reinforced responding below baseline and vehicle treated levels (Fig. 2). Importantly, the lack of an effect of CP-154,526 on water- (and sucrose-) reinforced lever pressing by mice not experiencing interruptions of operant procedures suggests that CRF-1 receptor signaling selectively modulates the expression of strengthened reinforced behaviors resulting from interruptions of procedures, and not reinforced behavior in general. Because the overall rate of reinforced responding in the first study (Figs. 1 and 2) appeared to be low, we determined if low operant behavior may have been caused by the stress associated with repeated daily i.p. injections. Consistent with this hypothesis, mice training to lever press for ethanol or water in the absence of daily i.p. injections showed noticeably greater levels of ethanol- and water-reinforced behavior (Fig. 4). In fact, following the single interruption from operant procedures, mice self-administered enough ethanol (approximately 65 lever presses over the 2-hour session) to consume approximately 2.0 g ethanol per kg. Based on published data that examined 2-hour ethanol self-administration by C57BL/6J mice (Middaugh et al. 2000b), mice that drank about 2.0 g/kg (as we found here) achieved blood ethanol levels of approximately 60-65 mg%. Thus, it would seem possible to achieve pharmacologically relevant blood ethanol levels in mice simply by interrupting daily operant self-administration procedures.

As noted above, we found that the magnitude and duration of the strengthened ethanol-reinforced lever pressing increased following multiple interruptions of operant procedures. At first glance, these observations appear to be consistent with previous studies that show an enhancement of the ADE following multiple cycles of access and deprivation in rats (Bell et al. 2004; McKinzie et al. 1998; Rodd et al. 2003; Rodd-Henricks et al. 2001; 2002a; b). However, it is questionable whether the present results reflect an ADE. Interruptions of operant procedures induce significant increases of reinforced lever pressing, whether the reinforcer was ethanol or

water. The ADE is typically defined as not only an increase in the absolute amount of ethanol consumed but also as an increase in the ratio of ethanol intake relative to total fluid consumed. The non-specific effect of interruptions of operant procedures on ethanol- and water-reinforced responding suggests that there may be an alternative explanation for the present data.

An interesting explanation consistent with the current data set is that the incentive motivational state elicited by the stimuli associated with the operant procedure (e.g., the various conditioned stimuli (CSs) within the operant chambers) strengthens during breaks, similar to a phenomenon that has been labeled "incubation" (Lu et al. 2004b). The increased incentive motivation elicited by these stimuli, in turn, promotes increased levels of responding once the mice are returned to the operant chambers. Viewed this way, it is the deprivation from the environmental CSs associated with the conditioning procedure, rather than deprivation from the reinforcer, that strengthens responding. In fact, in the present study mice are never deprived of water (as they have access to water in their home cage) yet they showed increased water-reinforced lever pressing after 4-days way from the operant procedures (Figs. 1, 2, and 5). Thus, it may be more appropriate to describe the current data set as reflecting an environmental deprivation effect (EDE) rather than an ADE. It is important to point out that increased lever pressing following interruptions of operant procedures is not likely caused by general behavioral arousal as interruptions did not alter responding to an inactive lever (Fig. 5). Thus, the EDE is specific to reinforced behaviors.

There are similarities between the EDE and the incubation phenomenon. For example, reinstatement of cocaine-seeking behavior induced by re-exposure to cocaine-associated CSs progressively increases in magnitude as the time interval between drug/CS removal and reinstatement testing increases (Grimm et al. 2001). Interestingly, time-dependent strengthening of CS-induced reinstatement of sucrose-reinforced responding has also been reported (Grimm et al. 2005; Grimm et al. 2002), indicating that this phenomenon is not specific to drugs of abuse

but can be observed with natural reinforcers (as we have noted here). Importantly, previous observations support the idea that it is the incentive motivational value of the CSs, rather than the reinforcing value of the drug/sucrose, that increases in a time-dependent manner. Thus, animals still show a time-dependent increase of CS-induced reinstatement of sucrose responding even when given *ad libitum* access to sucrose in their homecages (Grimm et al. 2005). On the other hand, there is no time-dependent increase in the ability of priming injections of cocaine to reinstate cocaine seeking behavior (Lu et al. 2004a).

Previous data have implicated CRF receptor signaling in the modulation of neurobiological responses to ethanol (Breese et al. 2004; Knapp et al. 2004; Le et al. 2000; Liu and Weiss 2002; Merlo Pich et al. 1995; Overstreet et al. 2004; Rassnick et al. 1993; Stewart 2004; Valdez et al. 2002). While the present data set do not allow us to infer a role of the CRF system in the expression of the ADE, we have found novel evidence that an increase in the ability of environmental CSs to motivate ethanol- (and water-) reinforced lever presses, resulting from time away from operant procedures (i.e., environmental deprivation), is modulated by CRF-1 receptor signaling. Although the neurochemical substrate underlying time-dependent increases of CS-induced drug-seeking behavior remains largely unknown, recent evidence suggests that this phenomenon is modulated by glutamate 2/3 receptor signaling within the central nucleus of the amygdala (Lu et al. Epub ahead of print). Interestingly, the glutamate system has also been implicated in the ADE (Backstrom et al. 2004; Holter and Spanagel 1999; Sanchis-Segura et al. 2006; Spanagel et al. 1996; Vengeliene et al. 2005). Our data suggest that CRF-1 receptor signaling is also involved in modulating the time-dependent increase of the incentive motivation associated with environmental CSs following EDE procedures.

In summary, we provide evidence indicating that interruptions of operant procedures increases the strength of ethanol- and water-reinforced lever pressing and that this phenomenon is modulated by CRF-1 receptor signaling. The observation that interruptions of

operant procedures augments lever presses reinforced with water indicates that the present data set may not reflect an ADE. The results are best explained by a time-dependent increase of the incentive motivation elicited by contextual conditioned stimuli as a result of deprivation from the conditioning environment. Thus, it may be more appropriate to describe the current data set as reflecting an "environmental deprivation effect" rather than an ADE. We suggest that it is critical to control for the possibility of an EDE when studying the ADE, particularly if ethanol consumption occurs in a environment other than the homecage as with operant procedures.

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FIGURE CAPTIONS

Fig. 1 Operant lever responses for 10% (v/v) ethanol (a) and water (b) during the 2-hour test sessions following the first 4-day break from operant procedures (Break 1) and after the fourth 4-day break (Break 4). Consumption of 10% (v/v) ethanol (g/kg) (c) and water (ml/kg) (d) during the 2-hour test sessions following breaks 1 and 4. Baseline (BL) refers to the average of the last three sessions before the first break. All values are means $\pm$ SEM. *$p < 0.05$ relative to baseline measures.

Fig. 2 Operant lever responses for 10% (v/v) ethanol (a) and water (b) during the 2-hour test immediately following a 4-day break from operant procedures. Mice were given an intraperitoneal (i.p.) injection of the CRF-1 receptor antagonist CP-154,526 (0, 10, 20 mg/kg) 30-minutes before testing. Baseline (BL) refers to the average of the last three sessions before the first break. All values are means $\pm$ SEM. *$p < 0.05$ relative to baseline measures.

Fig. 3 Operant lever responses for 10% (v/v) sucrose (a) and water (b) during the 2-hour test. Mice were given an intraperitoneal (i.p.) injection of the CRF-1 receptor antagonist CP-154,526 (0, 10 mg/kg) 30-minutes before testing. All values are means $\pm$ SEM. *$p < 0.05$ relative to baseline measures.

Fig. 4 Operant lever responses for 10% (v/v) ethanol (a) and water (b) during the 2-hour test sessions following a 4-day break from operant procedures in mice that did not receive habituation injections. Consumption of 10% (v/v) ethanol (g/kg) (c) and water (ml/kg) (d) during the 2-hour test sessions following the break. Baseline (BL) refers to the average of the last three sessions before the 4-day break were introduced. All values are means $\pm$ SEM. *$p < 0.05$ relative to baseline measures.

Fig. 5 Operant lever responses for water (a) and the inactive lever (b) during the 2-hour test sessions following the 4-day break away from operant procedures. Baseline (BL) refers to the average of the last three sessions before the break. All values are means $\pm$ SEM. *$p < 0.05$ relative to baseline measures.
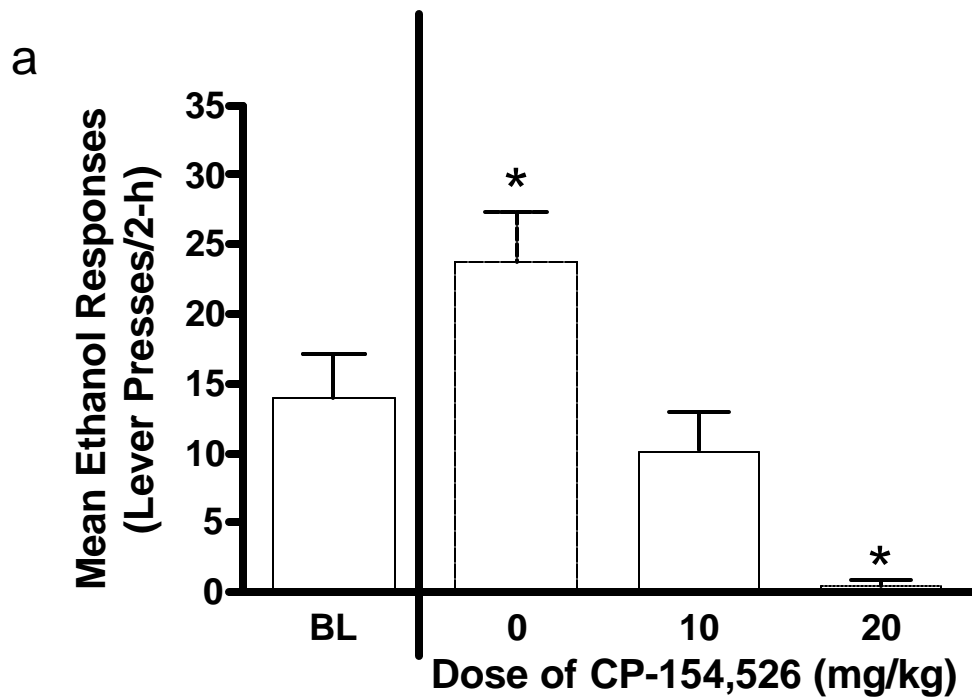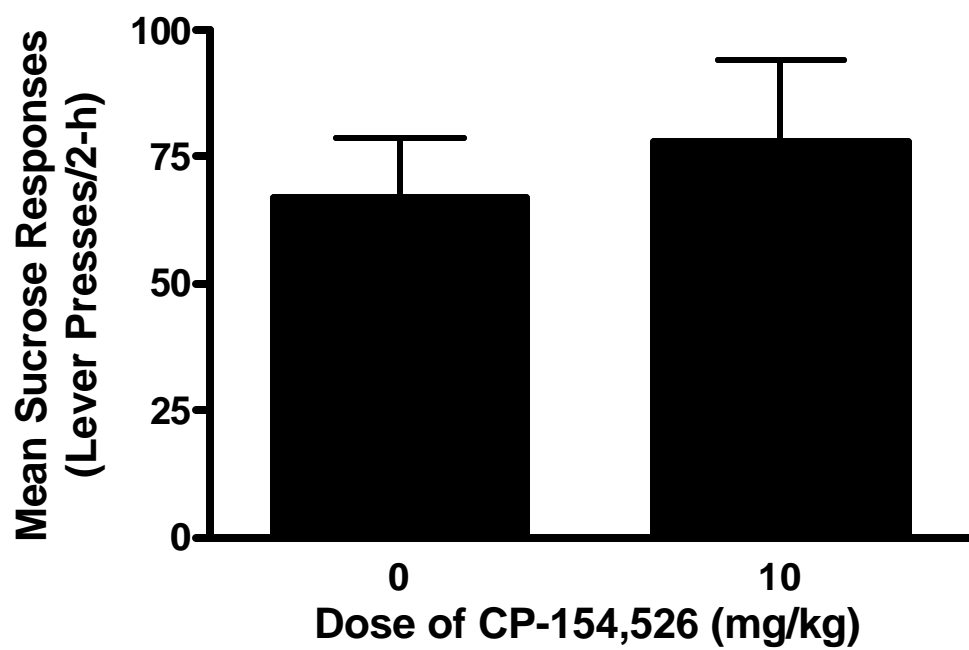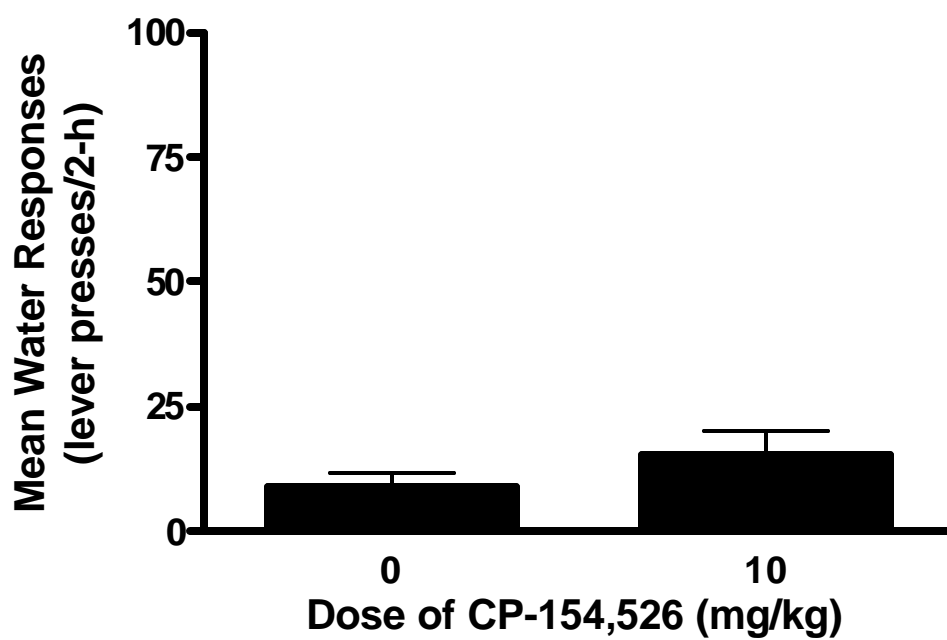
Figure1

a

b

c

d

a

b
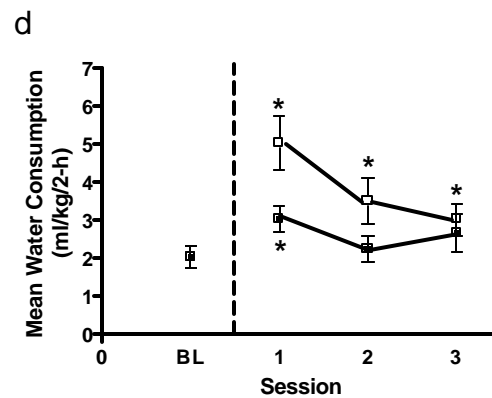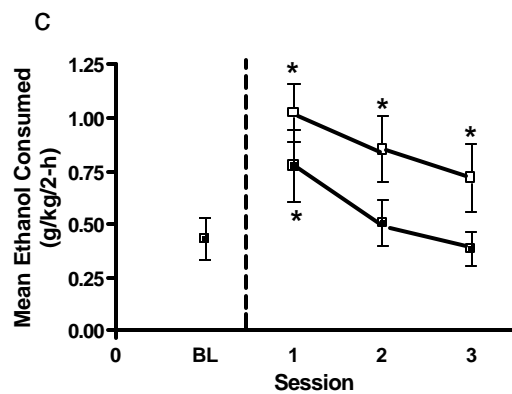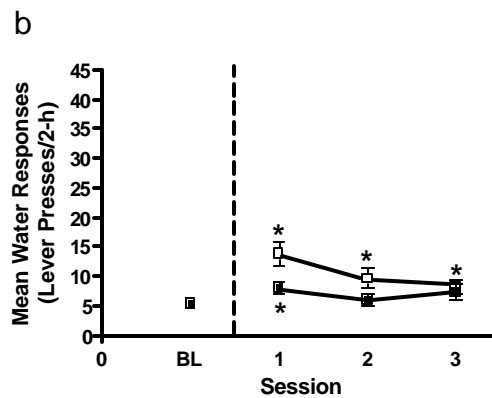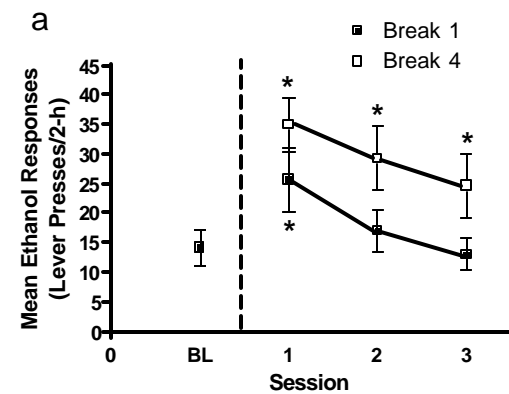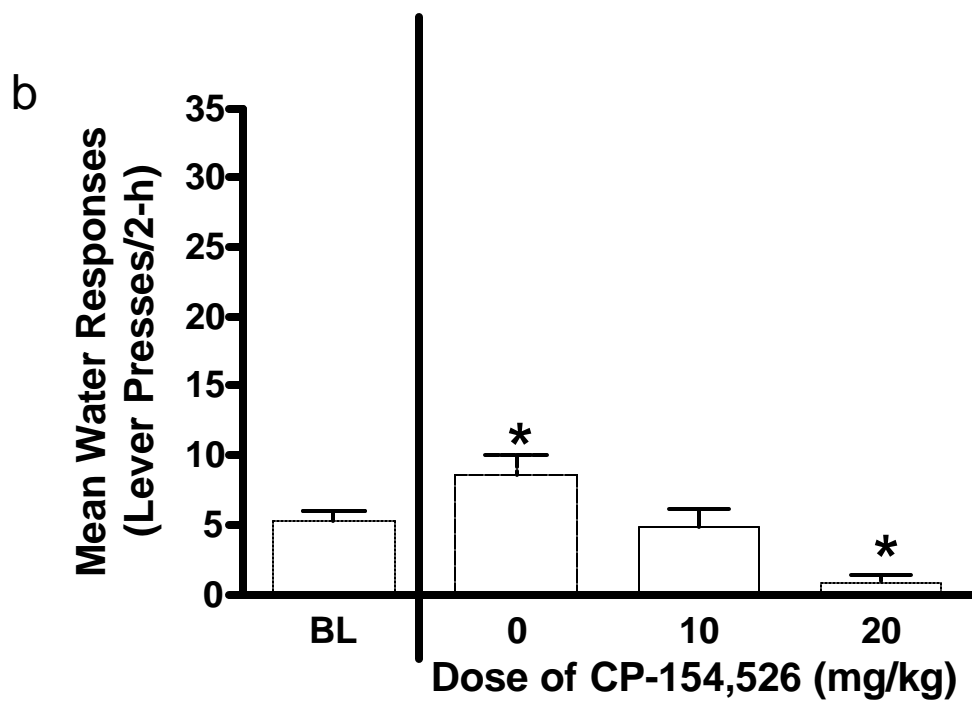
Figure2

a

b

Figure 3

Figure 4

Figure 5